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Trace Analysis of Zn(II), Be(II), and Bi(III) by Enzyme-Catalyzed Chemiluminescence

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A novel technique for the trace analysis of metal ions Zn(II), Be(II), and Bi(III) in bulk solutions is discussed. This technique involves the generation of a chemiluminescence signal from alkaline phosphatase catalyzed hydrolysis of a phosphate derivative of 1,2-dioxetane. Zn(II) can be determined by two methods, reactivation of the alkaline phosphatase apoenzyme and inhibition of the native enzyme. Be(II) and Bi(III) can be determined quantitatively by inhibition of the native enzyme. Subppb to ppm level detection of Zn(II), Be(II), and Bi(III) has been achieved. Initial studies with mixed metals are also reported. The technique described is rapid and sensitive and can be readily applied to the microassay of heavy metal ions.

Activation and inhibition of enzymes by inorganic species as a means of selective and sensitive inorganic trace analysis has been a focus of study for the last several years.[1-10] Microassay for heavy metal ions present in samples such as biofluids, fermentation broth, river water, and waste waters provides useful information not only in the diagnosis of disease but also in quality control and control of environmental contamination.

Metalloenzymes like alkaline phosphatase and carbonic anhydrase require heavy metal ions for their catalytic activity. These ions are generally coordinated in specific locations in the active sites of the enzyme and function as a cofactor in the catalytic reaction. Removing the metal ions from the enzyme with strong chelating agents results in the formation of the corresponding apoenzyme lacking enzyme activity. By reexposure to the metal ion, the apoenzyme can be reversibly activated. Therefore, the amount of the metal complexed in the active site of the enzyme should be directly related to the enzyme activity induced by the coordination. Thus, enzyme activity would be proportional to the added amount of the metal ions. The trace metal ion content in a solution can thus be evaluated by measuring the increase in activity due to reactivation of the apoenzyme. However, some metal ions are known to inhibit the activity of the native enzyme. An example of this is alkaline phosphatase, a dimeric enzyme which contains four Zn(II) ions, two per monomer active site. While apoalkaline phosphatase can be reactivated with Zn(II) ions,[1-3] Be(II), Zn(II), and Bi(III) are known to be potent inhibitors of the native alkaline phosphatase.[4-7] It is thus also possible to develop sensitive methods for the determination of Be(II), Zn(II), and Bi(III) concentrations on the basis of their inhibitory effects on the native enzyme.

Techniques based on optical and electrochemical methods of signal transduction have been devised to detect Zn(II) in the nanomolar range.[7-10] In this paper we report a novel technique for the determination of Zn(II) ions in trace levels (ppb range) by alkaline phosphatase regeneration and in the ppb to ppm range by enzyme inhibition. In addition to Zn(II), Be(II) and Bi(III) also have been determined quantitatively on the basis of their inhibitory effect on alkaline phosphatase. The technique involves the detection of the chemiluminescence signal generated by the action of alkaline phosphatase on 3-{spiro(4-methoxy-1,2-dioxetane-3-2′-tricyclo[3.3.1.1]chlorodecan-4-yl)}phenyl phosphate (CSPD) in the presence and absence of metal ions. The effects of various cations and anions on the enzyme activity have been studied. Because of in situ light generation in the reaction mixture, assembly and alignment of optical components become simple.

EXPERIMENTAL SECTION

Materials. Alkaline phosphatase (3 units/mg) from bovine calf intestine (EC 3.1.3.1), Tris-HCl, ammonium sulfate (enzyme grade), zinc sulfate, and *p*-nitrophenyl phosphate were supplied by Sigma (St. Louis, MO) and sodium acetate, sodium chloride, hydrochloric acid (metal free), and magnesium chloride were purchased from Fisher Scientific (Fair Lawn, NJ). Beryllium sulfate, bismuth nitrate, sodium fluoride, and acetylacetone were purchased from Aldrich Chemical Co. (Milwaukee, WI). Diethylamine (DEA) and CSPD were supplied as a part of a Southern-Light Chemiluminescent Detection System by Tropix, Inc. (Bedford, MA). CSPD was supplied as a 25 mM aqueous solution. Sapphire, a luminescent amplifying material (enhancer), was also supplied by Tropix, Inc. Deionized and distilled water was used in all preparations. All chemicals were of maximum purity available and were used as received.

Methods. Apoenzyme was prepared by dialyzing 15–20 mL of alkaline phosphatase (10 mg/mL) against 2 L of 2 M ammonium sulfate, at pH 9.0. The dialysis was carried out at 4 °C for 24 h

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with two changes of dialysate. Ammonium sulfate was removed by dialysis against 2 L of 0.01 M Tris-HCl, 0.01 M sodium acetate, and 0.1 M sodium chloride at pH 9.0 with two changes of dialysate.[11] Enzyme activity was measured by following the hydrolysis of *p*-nitrophenyl phosphate in 0.2 M Tris-HCl, pH 8.0, buffer. The initial rate of formation of *p*-nitrophenol was recorded at 405 nm using a Perkin-Elmer Lambda 9 UV–vis–near-IR spectrophotometer. Zinc ion concentration in the enzyme and apoenzyme was checked by a direct current plasma (DCP) spectrometry (Model SMI III Spectrametrics Inc.).[12] For reconstitution of the apoenzyme, the dialyzed enzyme was incubated with different concentrations of Zn(II) for 2 min. Stock solutions of Zn(II) and other metals were prepared by dissolving the spectral grade salts in deionized distilled water. Further dilutions were made in DEA.

The assay buffer for chemiluminescence experiments was prepared weekly as recommended by the supplier, by dissolving DEA and $MgCl_2$ in deionized distilled water to final concentrations of 0.1 and 1.0 mM, respectively. The pH was adjusted to 10.0 by adding 0.1 M HCl (metal free). Stock substrate was prepared as a 10% solution of enhancer in DEA buffer. To 10 mL of this solution was added 2 μL of 25 mM CSPD solution. The stock solutions, stored at 4 °C, were brought to room temperature prior to the reaction. The reaction mixture was prepared in a glass test tube by first adding predetermined volumes of enzyme of known concentration and metal ion solution to buffer. The reaction was initiated by adding 5.0 mL of the substrate solution. The composition of the final reaction mixture was determined after extensive experimentation to minimize the amounts of enzyme and CSPD and to maximize the signal-to-noise ratio.

Determination of Zn(II) by Regeneration of Activity. A typical reaction mixture of 1.60 mL (for control) was prepared by mixing 0.5 mL of 0.4 mM CSPD solution, 1.05 mL of buffer, and 50 μL of enzyme solution (10 mg/mL). The reaction was initiated by adding 0.5 mL of 0.4 mM CSPD. Zn(II) determination was done by incubating 50 μL of apoenzyme with 50 μL of metal ion solution for 2 min, to which 1.0 mL of buffer and 0.5 mL of 0.4 mM CSPD were added.

Determination of Zn(II), Be(II), and Bi(III) by Inhibition of Activity. The reaction mixture of 0.350 mL was prepared by mixing 0.125 mL of 5.0 μM CSPD solution, 0.125 mL of metal solution, and 0.1 mL of enzyme solution (1 mg/mL, activity 3 units/mL). For the control, 0.125 mL of assay buffer was added instead of metal solution. Metal ion concentration was varied either by adding a fixed volume of diluted metal solution or by adding different volumes of the stock metal solution and making up the reaction mixture volume to 0.350 mL with buffer.

Determination of Zn(II) in the Presence of Be(II) by Selective Masking. The reaction mixture of 0.7 mL was prepared by mixing 0.25 mL of 5.0 μM CSPD solution, 0.125 mL of metal ion solution, 0.125 mL of masking agent [acetylacetone 0.02% v/v in 0.1 M NaF for determination of Zn(II)], and 0.2 mL of enzyme solution (at 3 units/mL). The blanks/control references were obtained by replacing the metal ion solution or masking agent by assay buffer.

A photomultiplier tube (Hamamatsu, Model R 943-02; photocathode material, GaAs(Cs); window, fused silica; wavelength range, 160–930 nm), an amplifier, a photon counter, and a microcomputer are used to collect and process the data. The experimental setup was described in greater detail earlier.[13] All the reactions were carried out in bulk. The same test tube was used in a given set of reactions to eliminate the effect of tube geometry on the detected signal intensity. Less than 5 s elapsed between the initiation of the reaction and the start of data acquisition. The signal reached peak value in approximately 60–70 s. Initial slopes (slope of rate of product formation or slope of chemiluminescent photon detection) were calculated based on the first 20 data points collected in as many seconds.

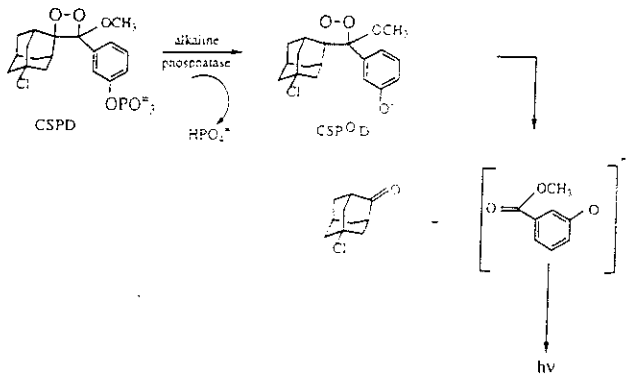

Figure 1. Representation of alkaline phosphatase catalyzed hydrolysis of CSPD.

RESULTS AND DISCUSSION

Typical chemiluminescence reactions for compounds such as lucigenin and luminol are characterized by the presence of an oxidant, a catalyst and alkali in the reaction mixture.[14] However, 1,2-dioxetanes such as CSPD release light in the absence of any oxidant. These compounds contain a powerful oxidizing potential built into the molecules with the presence of the inherently weak O–O bonds in highly strained four-membered rings.[15] Decomposition of dephosphorylated CSPD is a highly exothermic process, releasing enough energy to allow the excitation of products to the higher electronic emissive states.[16,17] Figure 1 illustrates the hydrolysis of CSPD catalyzed by alkaline phosphatase and subsequent photon emission. A kinetic expression presented elsewhere was derived for the rate of generation of chemiluminescence or from an experimental point of view, the rate of photon detection.[18] Typical postexperimental data processing involved conversion of a real-time curve of photon count rate versus time to a counts versus time curve by data integration. The slope of the curve in its linear portion was then computed and equated to the initial velocity of the reaction. The velocity of the reaction is the rate of chemiluminescence signal generation. For simplicity we measure the slope of the linear portion of the photon count per second versus time curve as a measure of enzyme activity. Maximum alkaline phosphatase activity for the substrate CSPD is observed at pH 10, similar to *p*-nitrophenyl

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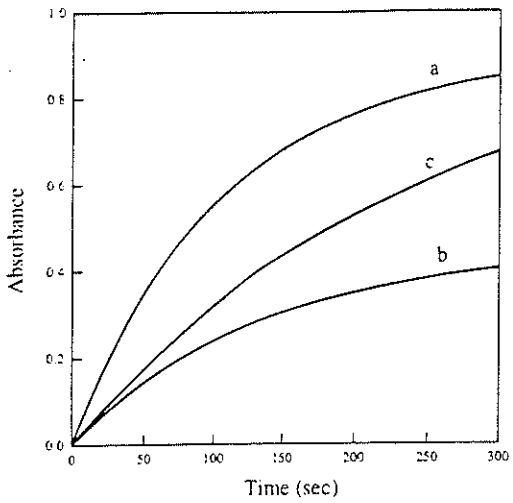

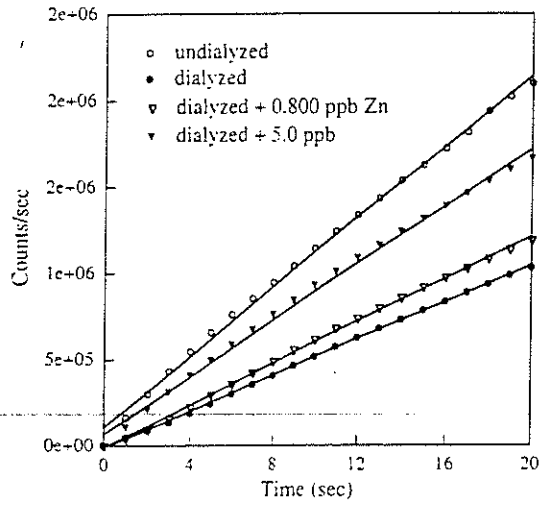

Figure 2. (a, top) Spectrophotometric response (405 nm) of the hydrolysis of *p*-nitrophenyl phosphate to 2.5 ppb of Zn(II): (a) undialyzed enzyme, (b) dialyzed enzyme, and (c) regenerated alkaline phosphatase. (b) Chemiluminescence curves for undialyzed, dialyzed, and regenerated alkaline phosphatase with 0.8 and 5.0 ppb Zn(II) ions.

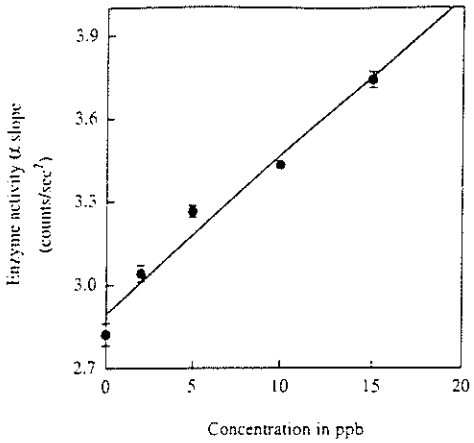

Figure 3. Activity regeneration in apoalkaline phosphatase as a function of solution Zn(II) concentration.

Table 1. Dependence of Reactivation of the Apoalkaline Phosphatase Enzyme Activity on Zn(II) Dynamic Range

enzyme activity (units/mL)	dynamic range (ppb)
22	0.5–5
30	2–15
293	10–50

phosphate (*p*NPP; pH 9.8).[3] Therefore, all the experiments were carried out at room temperature and pH 10, where the enzyme shows maximum activity. The enzyme activity can be expressed in terms of chemiluminescence signal strength as peak photon counts or initial slopes of counts per second versus time plot.

1. Zn(II) Detection by Signal Enhancement. Apoalkaline phosphatase exhibits a reduced catalytic effect on the hydrolysis of the substrate *p*NPP compared to the zinc-containing native enzyme.[3] This principle has been used to develop different techniques for the determination of zinc.[6,7] Figure 2a illustrates a typical spectrophotometric response of the hydrolysis of the substrate *p*NPP by undialyzed, dialyzed, and regenerated [by Zn(II)] enzyme, monitored at 405 nm. After adding Zn(II) ions, the apoalkaline phosphatase regains its activity, resulting in an increased rate of hydrolysis of *p*NPP. It is also evident from the figure that dialyzing the enzyme with $(NH_4)_2SO_4$ resulted in only 50% apoenzyme formation.

We observed a similar effect on the chemiluminescence generation rate from the dephosphorylation of CSPD. The reactions were carried out as described earlier with undialyzed and dialyzed enzyme and dialyzed enzyme incubated with different concentrations of Zn(II) ions. Data collection was continued for about 1 min, well past the linear portion of the counts per second versus time curve. In practice, the data points required to compute slopes could be collected within 20–25 s. The initial slopes were determined with data points collected during the first 20 s. DEA buffer in the reaction mixture plays a significant role of scavenging the inorganic phosphate, a potential enzyme inhibitor generated during the reaction. Inhibition rather than enhancement was observed when similar reactions were carried out without excess assay buffer solution. Figure 2b illustrates a set of chemiluminescence curves for undialyzed, dialyzed, and regenerated (by Zn(II)) enzyme. Figure 3 shows the calibration curve for Zn(II) ion. The error bars in the figures signify the standard deviation of five replicate measurements. Detection limit is the concentration of metal ions determined for three standard deviations above the mean of five replicate measurements of the zero standard (control, without any metal ion). A detection limit of 0.5 ppb was achieved with the chemiluminescence technique, which is an improvement over the earlier well-established colorimetric methods.[3] The upper limit (maximum concentration detected) of the dynamic range for the Zn(II) is set by the amount of zinc present in the undialyzed enzyme (source), the concentration of the enzyme used for analysis, and the efficiency of dialysis. Table 1 gives the dependence of the dynamic range on the protein concentration. As in the case of native enzyme, even apoenzyme regeneration is influenced by the presence of various metal ions. Table 2 depicts the influence of some known interfering metal ions on apoenzyme regeneration. The order of enhancement by these metal ions is Zn > Co > Cd > Ca > Mg > Ba.

2. Detection of Zinc, Beryllium, and Bismuth by Inhibition. A sample for metal ion detection often contains a variety of inorganic and organic ionic species in various concentrations. Therefore, different metal cations and anions were tested for their inhibitory effect on enzyme-catalyzed chemiluminescence; Be(II),

Table 2. Extent of Activity Regeneration in Apoalkaline Phosphatase by Different Divalent Metal Ions (at 10 ppb Concentration)

element	relative activity
dialyzed enzyme	1.0000
Cd^{2+}	1.1114 ± 0.0014
Zn^{2+}	1.2920 ± 0.0016
Co^{2+}	1.2515 ± 0.0003
Ba^{2+}	1.0093 ± 0.0002
Mg^{2+}	1.0198 ± 0.0011
Ca^{2+}	1.0329 ± 0.0001

Table 3. Inhibitory Effect of Various Metal Ions and Anions (at 200 ppm Concentration) on the Enzyme-Catalyzed Chemiluminescence Signal[a]

cations	relative activity	anions	relative activity
Na^{-}	1.0000	Cl^{-}	1.0000
Al^{3-}	0.7543 ± 0.0326	F^{-}	1.0248 ± 0.0002
Ca^{2-}	0.8245 ± 0.0486	EDTA	0.7256 ± 0.0215
Ba^{2-}	0.7319 ± 0.0047	SO_3^{-}	0.9753 ± 0.0025
Pb^{2-}	0.7407 ± 0.0086	CO_3^{-}	0.9322 ± 0.0088
Cu^{2-}	0.5884 ± 0.0123	NO_3^{-}	0.7120 ± 0.0020
Mg^{2-}	0.7649 ± 0.0075	PO_4^{-}	0.4705 ± 0.0035
Co^{2-}	0.9450 ± 0.0234	CH_3COO^{-}	0.9507 ± 0.0001
Ni^{2-}	0.9612 ± 0.0203		
Be^{2-}	0.0260 ± 0.0013		
Bi^{3-}	0.5154 ± 0.0170		
Zn^{2-}	0.3384 ± 0.0077		
Fe^{2-}	0.8417 ± 0.0916		
Cd^{2-}	0.8617 ± 0.0179		

[a] Enzyme activity measured relative to the effect of 200 ppm NaCl.

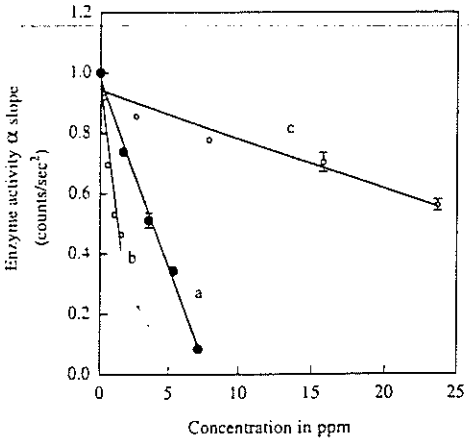

Figure 4. Inhibitory effect of (a) Zn(II), (b) Be(II), and (c) Bi(III) on chemiluminescence signal.

Zn(II), and Bi(III) had the largest inhibitory effect among metal ions evaluated along with phosphate anion (Table 3). The dependence of enzyme activity on these metal ions as a function of concentration is given in Figure 4. The experiments were done at a fixed concentration of the enzyme and CSPD. The metal ion concentration was varied to successively lower concentrations until the difference between the initial slope of the control (absence of any metal ion inhibitor) and one in the presence of metal ion was negligible (within three standard deviations of the control). From Table 3 and Figure 4, it is evident that the extent of inhibition is in the order Be(II) > Zn (II) > Bi(III). From the data for Zn(II), Be(II), and Bi(III), it is established that the chemiluminescence technique could be used to detect ppb to ppm levels of these metal

Table 4. Comparison of the Detection Limits for Different Metal Ions by Various Detection Techniques

technique	detection limit (ppm) Zn(II)	Be(II)	Bi(III)
current[a]	0.17	0.001	1.8
current[b]	0.0005		
fluorescence[5]		0.01	1.0
absorbance[3,4]	0.6	0.018	
electrochemical[8]	0.13		
chemiluminescence[10]	0.001		
absorbance[12]	0.006		

[a] Inhibition of native enzyme. [b] Activation of apoenzyme.

Table 5. Determination of Zn(II) in the Presence of Be(II) by Selective Masking of Be(II) Ions Using Acetylacetone and Sodium Fluoride[a]

Be(II) (ppb)	Zn(II) (ppm)	relative activity
0.0	0.0	1.0[b]
0.0	0.0	1.165 ± 0.016
8.928	0.0	0.279 ± 0.014[b]
8.928	0.0	0.916 ± 0.028
0.0	8.928	0.732 ± 0.009
8.928	8.928	0.719 ± 0.008
0.0	13.392	0.677 ± 0.018
8.928	13.392	0.663 ± 0.013
0.0	17.857	0.578 ± 0.004
8.928	17.857	0.571 ± 0.019

[a] Masking agent, 0.0003 M acetylacetone and 0.0178 M NaF (acetylacetone 0.02% v/v in 0.1 M NaF used in reaction mixture). Enzyme activity in reaction mixture is 0.857 unit. CSPD concentration in reaction mixture is 1.785 μM. [b] No masking agent used in these sets of experiments.

ions. The detection limits achieved by inhibition for Zn(II), Be(II), and Bi(III) were 0.170, 0.001, and 1.8 ppm, respectively. These detection limits are quite comparable and, in the case of Be, superior to those reported earlier by other techniques as shown in Table 4.

In the above case, the effective CSPD concentration in the reaction mixture was 1.785 μM, whereas in our earlier experiments, we used a CSPD concentration of 2.631 μM with the same enzyme concentration; the detection limits achieved by inhibition for Zn(II), Be(II), and Bi(III) were 0.360, 0.265, and 2.6 ppm, respectively. Hence, we believe that by lowering the enzyme and CSPD concentrations in the reaction mixture the detection limits can be lowered further for metal ion detection.

3. Selective Determination of Metal Ions Using Masking Agents. The determination of metal ions by inhibition of the enzyme can be made metal ion specific using different masking agents and by suitable sample pretreatment.[4,19] Table 5 illustrates the determination of Zn(II) in the presence of the potent inhibitor Be(II) by selective masking of Be(II) using acetylacetone and sodium fluoride. Acetylacetone effectively masks Ag, Ni, Co, and Cu ions along with Be, and NaF masks the effect of Al, Ca, Sr, and Mn if these interfering metal ions are present in the sample.[4,19] In these experiments, different controls were performed with and without masking agents and with either or both of the metal ions present. It is quite evident from Table 5 that the inhibitory effects of Zn(II) in the presence and absence of Be(II) are the same due

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to the masking effect of the agent. Moreover, masking agent alone had some enhancement effect on relative activity which could be attributed to the presence of fluoride as seen before in Table 5.

Similarly, in the selective determination of Be(II) masking agents like thioglycolic acid, unithiol (2,3-dimercaptopropane-1-sulfonic acid), and diethyldithiocarbamate can be used to masks the interference from the metal ions like Zn, Hg, As, Cd, Pb, Bi, Sb, Cu, and Ag.[19]

CONCLUSIONS

A novel technique for the determination of trace level of Zn(II), Be(II), and Bi(III) is described. Alkaline phosphatase catalyzed chemiluminescence is used to establish calibration curves for these metal ions. In addition to rapid and sensitive detection, the technique uses a simplified optical experimental setup. A 0.17 ppm, 1 ppb, and 1.8 ppm level of detection was achieved for Zn(II), Be(II), and Bi(III), respectively, by the enzyme inhibition method. Zn(II) was also detected at 0.5 ppb by the apoenzyme regeneration method. Selective determination of Zn(II) in the presence of Be(II) was demonstrated by masking the interfering ions with acetylacetone and sodium fluoride. Other metal ions can be selectively determined by the proper choice of masking agents to suppress the interfering ions.

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